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Carnosic acid, a new class of lipid absorption inhibitor from sage

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Abstract—The methanolic extract from the leaves of *Salvia officinalis* L. (sage) showed significant inhibitory effect on serum triglyceride elevation in olive oil-loaded mice (500 and 1000 mg/kg, p.o.) and inhibitory activity (IC_{50}: 94 μg/mL) against pancreatic lipase, which is participated in digestion of lipids. Through bioassay-guided separation using the inhibitory activity against pancreatic lipase activity, 4 abietan-type diterpenes (carnosic acid, carnosol, royleanonic acid, 7-methoxyrosmanol) and a triterpene (oleanolic acid) were isolated from the active fraction. Among these compounds, carnosic acid and carnosol substantially inhibited pancreatic lipase activity with IC_{50} values of 12 μg/mL (36 μM) and 4.4 μg/mL (13 μM), respectively. Carnosic acid significantly inhibited triglyceride elevation in olive oil-loaded mice at doses of 5–20 mg/kg (p.o.). However, other constituents (carnosol, royleanonic acid, oleanolic acid) did not show any effects at a dose of 200 mg/kg (p.o.). Furthermore, carnosic acid (20 mg/kg/day, p.o.) reduced the gain of body weight and the accumulation of epididymal fat weight in high fat diet-fed mice after 14 days.

1. Introduction

Overweight and obesity are recognized to be important risk factors for type II diabetes, dyslipidemia, hypertension and so on.[1] To regulate fat absorption is an effective way to reduce body weight and obesity. Pancreatic lipase is well known to play an important role in lipid digestion, and a strong pancreatic lipase inhibitor, orlistat, is clinically used for obesity by reducing the energy from the diet.[2] In the course of our studies on anti-obese constituents from natural medicines,[3] we investigated the inhibitory activities of various herbal extracts against porcine pancreatic lipase activity. As a result, the methanolic (MeOH) extract from the leaves of *Salvia officinalis* L. substantially inhibited the pancreatic lipase activity, and it also suppressed serum triglyceride (TG) elevation in olive oil-loaded mice.

The Labiatae plant *S. officinalis* (sage, salvia), originated from Mediterranean area, has been cultivated in many countries. The leaves of this plant have been most commonly known not only as a culinary herb for flavoring and seasoning, but it has been also of great medicinal importance such as anti-lactation, antiinflammatiton, anti-sore throat, and anti-dyspepsia. In the previous studies, many compounds such as diterpenoids,[4] triterpenoids,[5] flavonoids,[6] and phenolic glycosides[7] were isolated from this plant. With regard to pharmacological studies of this herb, anti-oxidative activity,[8] HIV-1 reverse transcriptase-inhibitory activity,[9] anti-Alzheimer's disease,[10] and insulin-like activity[11] were reported. However, effects of this herb on pancreatic lipase activity and lipid digestion were not reported to date. In this paper, we describe the active constituents against pancreatic lipase activity and its anti-obese effects.

2. Results and discussion

2.1. Isolation of active constituents from the leaves of *S. officinalis*

The dried leaves of *S. officinalis* (480 g, cultivated in Albania, purchased from Tochimoto Tenkaido Co. Ltd, Osaka, Japan) were extracted with methanol (MeOH) under reflux. The MeOH extract (yield: 30.4% from the natural medicine) was partitioned into an ethyl acetate (EtOAc) and water mixture to give an EtOAc-soluble portion (16.1%) and an aqueous phase. The aqueous phase was further extracted with 1-butanol (1-BuOH) to give a 1-BuOH-soluble portion (3.4%) and a water-

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soluble portion (10.9%). The MeOH extract showed inhibitory activity against porcine pancreatic lipase activity (IC_{50} = 94 μg/mL), and it also suppressed serum TG elevation in olive oil-loaded mice at doses of 500 and 1000 mg/kg.

To clarify the active constituents in the MeOH extract, a bioassay-guided separation was performed using the inhibitory activity against pancreatic lipase. The EtOAc-soluble fraction (IC_{50} = 31 μg/mL) showed stronger inhibitory activity than the other two fractions (1-BuOH and H_2O-soluble fractions >300 μg/mL). Next, the EtOAc-soluble portion was subjected to silica gel column chromatography [*n*-hexane–AcOEt (10:1→3:1→1:1, v/v)→$CHCl_3$–MeOH (10:1, v/v)→MeOH] to give 4 fractions (fr.1–fr.4). The active fractions 2 and 3, which showed strong inhibition against lipase activity by 94% and 88% at 100 μg/mL, were subjected to ODS [MeOH–H_2O (60:40→90:10, v/v)] column chromatography and finally HPLC [MeOH–H_2O (85:15, v/v)] to give 4 abietan-type diterpenes [carnosic acid (**1**,[12] yield 0.29% from this herb), carnosol (**2**,[13] 0.24%), royleanonic acid (**3**,[14] 0.011%), and 7-methoxyrosmanol (**4**,[13] 0.081%)] and a triterpene [oleanolic acid (**5**, 0.47%)] as shown in Chart 1. These 5 compounds were identified by comparison of their physical data ($[\alpha]_D$, IR, ^1H NMR, ^{13}C NMR, MS) with reported values or with those of authentic sample of **5**.

The effects of the isolated compounds (**1**–**5**) on lipase activity were examined. Among them, carnosic acid (**1**) and carnosol (**2**) substantially inhibited the activity with IC_{50} values of 12 μg/mL (36 μM) and 4.4 μg/mL (13 μM), respectively (Table 1), although their activities were weaker than that of orlistat [IC_{50} = 1.5 ng/mL (3.0 nM)].

Next, we examined the effects of these compounds on TG elevation in olive oil-loaded mice by oral administration. As shown in Table 2, carnosic acid (**1**) dose-dependently suppressed serum TG elevation. However, different from the results in vitro, the inhibitory effect of **1** was equivalent to that of orlistat. Although, carnosol (**2**) showed stronger lipase inhibitory activity than **1**, compound **2** as well as **4** and **5** did not suppressed TG elevation at a dose of 200 mg/kg in vivo. Thorsen et al. reported carnosol (**2**) was unstable in various solvent, but carnosic acid (**1**) was stable.[15] The lacking effect of **2** in vivo may be due to its unstability. These results suggest that compound **1** is the most effective compound among the isolated constituents and its suppressive effects on serum TG elevation in olive oil-loaded mice is, at least in part, dependent on the inhibitory activity against pancreatic lipase. Detailed investigations of anti-hyperlipidemic effects of **1** including the lacking effects of **2** in vivo need to be made.

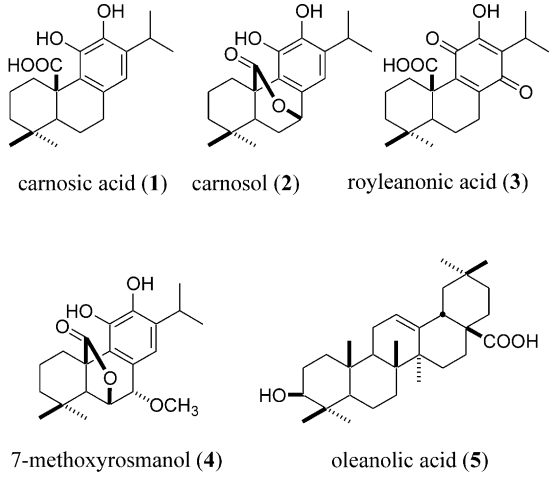

Chart 1. Isolated compounds from the leaves of *S. officinalis*.

To examine the type of inhibition of **1** against porcine pancreatic lipase, the enzyme was incubated with increasing concentrations of the substrate (2,3-dimercaptopropan-1-ol tributyrate; 0.03–0.20 mM). The result plotted according to Lineweaver–Burk revealed a fully competitive type of inhibition on porcine pancreatic lipase and K_i value of **1** on the enzyme was 5.4 μg/mL (16.1 μM).

Finally, effects of **1** on the weight of epididymal fat as a marker of visceral fat accumulation were examined in high fat diet-fed mice, since the accumulation of the visceral fat is recognized the major factor which make the life-style related diseases, such as type II diabetes, hypertension, hyperlipidemia, and so on.

As shown in Table 3, the high fat diet feeding for 14 days did not significant increase in body weight of mice compared to that of normal group, but significantly increased in the weight of epidydimal fat pad. Compound **1** significantly reduced the gain of the body weight at a dose of 20 mg/kg/day for 14 days. In addition, the increase in weight of epidydimal fat pad was significantly suppressed by oral administration of **1** at doses of 5–20 mg/kg/day. In our conditions, no significant difference was observed in serum TG levels between the control and normal groups. However, serum TG levels was significantly reduced after administration of **1** (10 mg/kg/day) for 14 days.

In conclusion, we isolated abietan-type diterpene constituents [carnosic acid (**1**), carnosol (**2**), royleanonic acid (**3**), and 7-methoxyrosmanol (**4**)] as a new class of pancreatic lipase inhibitor from the leaves of *S. officinalis*. Among them, **1** suppressed serum TG elevation in olive oil-loaded mice and increase in weight of epididymal fat in high fat diet-fed mice. Therefore, **1** could be effective for cure of obese patients due to over intake of high fat diet.

Table 1. Inhibitory effects of constituents (**1**–**5**) from the leaves of *S. officinalis* against porcine pancreatic lipase

Sample	Inhibition (%)						IC_{50}
Conc. (μg/mL)	1	3	10	30	100	300	(μg/mL)
Carnosic acid (**1**)	4	12	36	76	96	—	12
Carnosol (**2**)	8	41	76	96	99	—	4.4
Royleanonic acid (**3**)	4	12	19	32	62	95	35
7-Methoxyrosmanol (**4**)	11	9	23	46	77	102	32
Oleanolic acid (**5**)	1	5	−5	18	68	78	83

An inhibitory test for pancreatic lipase activity was determined using porcine pancreatic lipase and 2,3-dimercaptopropan-1-ol tributyrate (final conc.: 2.0 mM) as a substrate. Each experiment was done in duplicate.

Table 2. Effects of the MeOH extract and constituents (**1**, **2**, **4**, **5**) from the leaves of *S. officinalis* on serum TG elevation in olive oil-loaded mice

Treatment	Dose (mg/kg, p.o.)	*N*	Serum TG (mg/100 mL) 2 h	4 h	6 h
Normal (water)	—	6	169±19**	114±6**	95±12**
Control (olive oil)	—	6	626±35	448±54	336±66
MeOH extract	500	6	335±71**	341±49	323±37
	1000	6	177±24**	289±32	299±44
Normal (water)	—	6	169±18**	118±11**	109±8**
Control (olive oil)	—	6	571±61	454±37	194±18
Carnosic acid (**1**)	5	6	316±40**	288±34**	143±16**
	10	6	390±39*	239±19**	112±16**
	20	6	220±25**	157±25**	85±9**
Normal (water)	—	6	181±18**	—	—
Control (olive oil)	—	7	587±43	—	—
Carnosol (**2**)	200	7	514±36	—	—
7-Methoxyrosmanol (**4**)	200	7	438±51	—	—
Oleanolic acid (**5**)	200	7	528±76	—	—
Normal (water)	—	5	172±16**	125±8**	97±11**
Control (olive oil)	—	7	543±31	405±37	258±23
Orlistat	5	5	317±89**	255±46**	145±35**
	10	5	237±34**	208±14**	101±6**
	20	5	177±15**	157±19**	103±7**

Values represent the means±SEM. Significantly different from control, *$p<0.05$, **$p<0.01$.

Table 3. Effect of carnosic acid (**1**) on weights of body and epididymal fat pad and serum TG levels after administration for 14 days in high fat diet-fed mice

Groups	Dose (mg/kg/day)	Body Weight (g) 1st day	7th day	14th day	Serum TG (mg/100 mL) 1st day	7th day	14th day	Epididymal fat pad (mg/mouse) 14th day
Normal (MF chow)	—	25.6±0.2	31.5±0.3	36.4±0.9	98±10	118±14	118±11	839±71**
Control (40% fat)	—	25.5±0.2	32.2±0.3	37.0±0.4	114±12	94±10	126±14	1472±106
Carnosic acid (**1**)	5	25.6±0.2	32.0±0.6	36.3±0.8	101±9	99±7	116±16	1024±109**
	10	25.6±0.2	32.4±0.8	36.4±1.2	103±10	85±11	78±4*	1018±88**
	20	25.5±0.3	30.3±1.0	34.2±0.6*	104±8	96±14	94±8	1005±49**

Values represent the means±SEM of 5 mice. Significantly different from control, *$p<0.05$, **$p<0.01$.

3. Bioassay methods

3.1. Effects on pancreatic lipase activity

An inhibitory test for pancreatic lipase activity was determined using a commercial kit [Lipase Kit S; substrate: 2,3-dimercaptopropan-1-ol tributyrate, Dainippon Pharmaceutical, Osaka, Japan] and porcine pancreatic lipase (L3126 Type II, Sigma-Aldrich, St. Louis, MO). Briefly, the reaction mixture containing test sample solution in DMSO (25 μL), the color reagent (390 μL), the enzyme solution (2.2 units/mL, 25 μL), and the esterase inhibitor (10 μL) was pre-incubated at 30 °C for 5 min, then the substrate solution (20 mM, 50 μL) was added to start the reaction and incubated for 30 min according to manufacturer's instructions. Each experiment was done in duplicate and the IC_{50} value was determined graphically by a plot of percent inhibitions versus log concentrations of the test sample. For kinetic analysis of porcine pancreatic lipase by carnosic acid (**1**), the enzyme and **1** (10 μg/mL) were incubated with increasing concentrations of the substrate (0.03–0.20 mM).

3.2. Effects on serum TG elevation in olive oil-loaded mice

Male ddY mice (6 w) was fasted for 20 h, and the test sample suspended in 5% acacia solution was given orally. Thirty min later, olive oil (Wako Pure Chemical Co. Ltd, Osaka, Japan) was given (5 mL/kg, p.o.). Blood samples were collected from the infraorbital venous plexus at 2, 4, and 6 h after loading of olive oil. Serum TG levels was determined using a commercial kit (Triglyceride G Test Wako; Wako Pure Chemical Co. Ltd, Osaka, Japan).

3.3. Effect on high fat diet-fed mice

High fat diet was prepared as follows. The visceral adipose tissue of cow was heated and melted by microwave oven. Obtained fat was added to the MF chow (Oriental Yeast Co. Ltd, Osaka, Japan) and mixed in the ratio of the fat was 40% (w/w).

Male ddY mice (6 w) were housed in this high fat diet for 14 days. Carnosic acid (**1**) suspended in 5% acacia

solution was given orally once a day at 16:00–17:00. Body weight of animals was weighed and recorded everyday. The mice were fasted for 20 h before 1, 7, and 14th day, and blood was collected from infraorbital venous plexus under ether anesthesia. Serum TG levels were determined using a commercial kit (Triglyceride G Test Wako). After administration for 14 days, mice were killed by cervical dislocation under ether anesthesia, and the epididymal fat pads were removed and weighed.

3.4. Statistics

Results were expressed as means ± SEM. Statistical significance was assessed by one-way analysis of variance followed by Dunnett's test. Probability (p) values less than 0.05 were considered significant.

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